



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

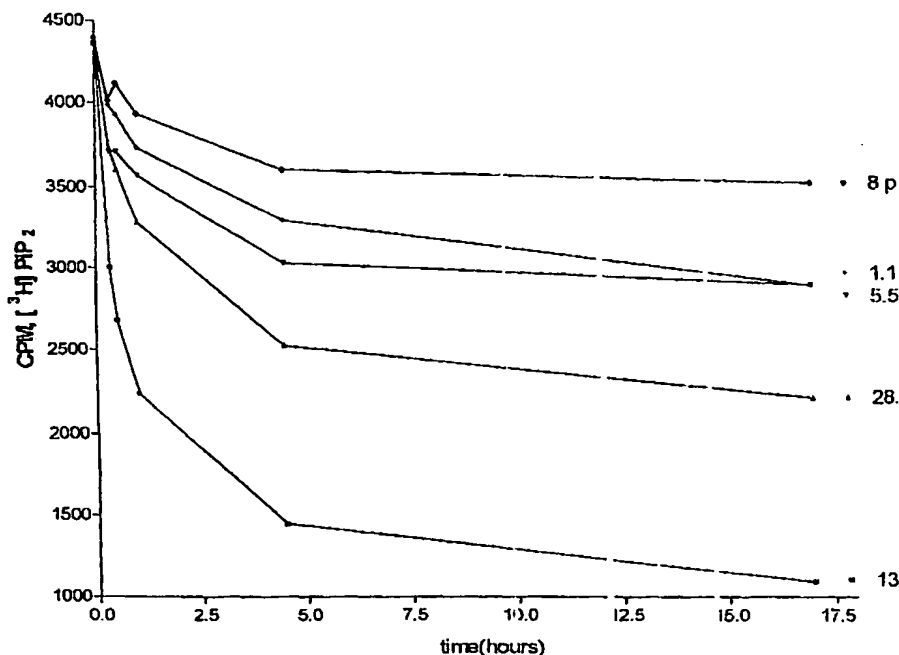
(51) International Patent Classification ⁶ : C12Q 1/00, G01N 33/53		A1	(11) International Publication Number: WO 99/32655
			(43) International Publication Date: 1 July 1999 (01.07.99)
(21) International Application Number: PCT/US98/26818			(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).
(22) International Filing Date: 17 December 1998 (17.12.98)			
(30) Priority Data: 60/068,244 19 December 1997 (19.12.97) US Not furnished 16 December 1998 (16.12.98) US			
(71) Applicant: NEN LIFE SCIENCE PRODUCTS, INC. [US/US]; 549 Albany Street, Boston, MA 02118 (US).			
(72) Inventors: KASILA, Patricia; 16 Kent Street, Windham, NH 03087 (US). MULLINAX, Thomas, R.; 178 Quinobequin Road, Newton, MA 02468 (US). BEMBENEK, Michael, E.; 243 Mill Street, Burlington, MA 01803 (US). BOBROW, Mark, N.; 11 Battle Green Road, Lexington, MA 02421 (US).			
(74) Agents: CITKOWSKI, Ronald, W. et al.; Gifford, Krass, Groh, Sprinkle, Anderson & Citkowski, P.C., Suite 400, 280 N. Old Woodward Avenue, Birmingham, MI 48009-5394 (US).			Published With international search report.

(54) Title: AN ENZYME ASSAY USING A SOLID PHASE SUBSTRATE

(57) Abstract

This invention relates to a hydrophobic layer formed on a solid support, the hydrophobic layer incorporating by hydrophobic interactions an amphipathic enzyme substrate labeled with a reporter on its hydrophilic region, and the use of the same to conduct various enzymes assays without extraction steps. The figure is a graph illustrating kinetic activity of various amounts of purified recombinant PLC gamma 1 on [3H]PIP coated 96-well phospholipid FlashPlate (tm).

Recombinant PLC γ 1 Activity
on [3H]PIP₂ Coated Phospholipid FlashPlate™



FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakhstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

AN ENZYME ASSAY USING A SOLID PHASE SUBSTRATE

Field of the Invention

This invention relates to a hydrophobic layer, such as a lipid layer formed on a solid support, the lipid layer incorporating by hydrophobic interactions an amphipathic enzyme substrate labeled with a reporter on its hydrophilic region, and
5 the use of the same to conduct various enzyme assays without extraction steps.

Background of the Invention

Because many intracellular and intercellular processes are membrane mediated, there has been a great deal of research on the reconstitution of biological
10 membranes as a method to study these processes. Since the original development of a procedure to form artificial planar phospholipid bilayer membranes (Mueller et al., 1962) and the demonstration of the fusion of vesicles that contain ion channels to planar membranes (Miller and Racker, 1976), studies have employed a variety of artificial membrane systems and various methods for studying
15 functional molecules that are incorporated in or bound to biological membranes (Hoekstra and Duzgunes, 1993). Some researchers have incorporated biotinylated lipids (e.g., see Wright and Huang, 1992) or functional enzymes (e.g., see Hianik et al., 1996) into artificial membranes, but the purpose has been to study properties of the membranes or the incorporated molecules, not to use the synthetic
20 membranes as a tool to study the properties of exogenous molecules as in the present invention. For a review of current techniques and research in this area (see Ottova and Tien, 1997).

Phospholipase C (PLC) is a generic name for enzymes that catalyze the hydrolysis of phosphoglycerides into diacylglycerols and phosphorylated alcohols

such as serine, choline, inositol, glycerol, or ethanolamine. For example, a specific phospholipase C hydrolyzes phosphatidylinositol-4-phosphate (PIP) or phosphatidylinositol-4,5-bisphosphate (PIP₂), resulting in each case in the formation of two second messengers: a hydrophobic diacylglycerol and a
5 hydrophilic inositol phosphate (IP₂ or IP₃ respectively). This hydrolysis can be monitored by a variety of methods using endogenously or exogenously labeled substrates.

De Vivo (1994) describes current methods used for measuring the hydrolysis of PIP₂. In the endogenous substrate approach, the cells of interest are
10 cultured in the presence of myo-[³H]-inositol. The cells convert inositol to phosphatidylinositol (PI) using phosphatidylinositol synthase, and the PI in turn is converted to PIP-[³H] and PIP₂-[³H] by phosphatidylinositol kinases. When the polyphosphoinositide pool is labeled to a steady state, the breakdown of PIP and PIP₂ is initiated by the addition to the cell culture medium of an appropriate
15 stimulatory factor (e.g., receptor agonists for intact cells or guanylyl nucleotides for permeabilized cells).

In the exogenous substrate approach, purified labeled phospholipids are used as the substrate. The phospholipids are mixed in the presence or absence of detergent and sonicated briefly on ice to prepare vesicles. Aliquots of the substrate
20 are mixed with a source of PLC (membranes or purified enzyme) and, often, G-protein subunits.

In both the endogenous and exogenous substrate approaches, solvent extraction is used to separate the hydrophilic reaction products from the hydrophobic substrate. Current methods for sphingomyelinase assays also require

solvent extraction steps. It would be desirable to eliminate the extraction step for environmental and health reasons. In addition, it is difficult to automate the extraction of large numbers of samples, as would be necessary for example in high throughput screening of drug candidates.

5

Summary of the Invention

The invention described here allows the quantitative or qualitative determination of the enzymatic activity of various enzymes without requiring an extraction step. As a substrate, the invention uses a solid support coated with a hydrophobic layer, such as an artificial lipid layer, incorporating by hydrophobic interactions an amphipathic enzyme substrate labeled so that a labeled hydrophilic fragment will be produced when the substrate is cleaved by a specific enzyme. The labeled hydrophilic fragment will migrate into the aqueous phase, thereby no longer being associated with the solid support, forming the basis for an assay of the activity of the enzyme. Selection of an appropriate labeled substrate allows the use of the invention with a variety of enzymes.

15

Brief Description of the Drawings

Other advantages of the present invention will be readily appreciated as the same becomes better understood by reference to the following detailed description when considered in connection with the accompanying drawings wherein:

20

Figure 1 is a graph illustrating kinetic activity of various amounts of purified recombinant PLC $\gamma 1$ on $[^3\text{H}]$ PIP₂ coated 96-well phospholipid FlashPlate™. Different concentrations of purified recombinant PLC were added to individual wells in 0.1 ml of 50 mM NaP_i, pH 6.7, 0.1 M NaCl, 0.1 mM CaCl₂, 0.1 mM EGTA and the release of radioactivity was monitored as a function of time

on a Packard TopCount™. (Microplate Scan System manufactured by the Packard Instrument Company of Meriden, Connecticut).

Figure 2A is a graph illustrating the effects of additives to PLC $\delta 1$ activity against [^3H] PIP₂ coated 96-well phospholipid FlashPlate™ wherein the effects of different concentrations of deoxycholate on the release of radioactivity as a function of time by 13 mU PLC in 0.2 ml HEPES-NaOH, pH 7.0, 0.14 M KCl, 0.1 mM CaCl₂, 0.1 mM EGTA, [deoxycholate]'s on a %(wt/vol) basis.

Figure 2B is a graph illustrating the effect of the addition of 0.2 mM spermine on 100 mU PLC activity in 0.2 ml 50 mM HEPES-NaOH, pH 6.7, 0.15 M KCl, 0.1 mM CaCl₂, 0.1 mM EGTA, 0.04% deoxycholate (wt/vol). The release of radioactivity was monitored on a Packard TopCount™.

Figure 3 is a graph illustrating the stimulation of PLC activity by addition of GTP γ S to crude HL60 cytosolic preparations by GTP γ S as measured on [^3H] PIP₂ coated 96-well phospholipid FlashPlates™. Various amounts of HL60 cytosol protein were added to individual wells containing 0.2 ml 50 mM HEPES-NaOH, pH 6.7, 0.15 M KCl, 0.1 mM CaCl₂, 0.1 mM EGTA, 0.04% deoxycholate (wt/vol) either in the presence or absence of 0.2 mM GTP γ S. The release of radioactivity was monitored on a Packard TopCount™.

Figure 4 is a graph illustrating the release of intracellular PLC activity from streptolysin S permeabilized A431 human carcinoma cells as measured on [^3H] PIP₂ coated 96-well phospholipid FlashPlate™. Aliquots of resuspended A431 cells were distributed into wells containing 50 mM HEPES-NaOH, pH 6.7, 0.15 M KCl, 0.1 mM CaCl₂, 0.1 mM EGTA, 0.04% deoxycholate (wt/vol), 10 units/ml

streptolysin S and the volume adjusted to 0.2 ml with reaction buffer. The release of radioactivity was monitored on a Packard TopCount™.

Detailed Description of the Invention

The unique feature of this invention is the use of a substrate that allows the study of enzyme activity in samples without the need to extract the reaction products. The invention can be used in either homogeneous or heterogeneous assay formats and can employ either radiometric or non-radiometric substrates. With appropriate substrate design, the present invention can be used to study a variety of enzymes, such as phospholipases, for which the assay methods otherwise require extraction steps. Substrates suitable for use in the invention include, but are not limited to phospholipids, sphingolipids, and any other amphipathic molecules, such as glycosyldiacylglycerols, ceramides, gangliosides, and complex phospholipids like cardiolipin. The enzyme used can be purified, semi-purified or a crude extract.

An amphipathic enzyme substrate labeled on a polar moiety is incorporated by hydrophobic interactions into any suitable hydrophobic layer, such as a lipid layer, that is formed on a solid support and is retained by hydrophobic interaction and/or by covalent attachment. A suitable hydrophobic layer is one that incorporates a component that binds the amphipathic substrate by hydrophobic interaction. The particular substrate to use is designed or selected for its susceptibility to the action of the enzyme of interest and an appropriate location for the label. Suitable reporter group(s) used to label the substrate include, but are not limited to radioactive isotopes, enzymes, fluorogenic, colorimetric, magnetic,

chemiluminescent or electrochemical materials or a member of a specific binding pair.

Suitable solid phase supports include but are not limited to synthetic polymer supports such as polystyrene, polypropylene, substituted polystyrene, e.g.,
5 laminated or carboxylated—polystyrene;—polyacrylamides; polyamides; polyvinylchloride, etc.; glass beads; agarose; nitrocellulose; nylon; polyvinylidenedifluoride; surface-modified nylon, etc. Homogeneous assay methods for phospholipases employing a 96-well FlashPlate as the solid support have been shown to work well. (A FlashPlate is a 96-well white polystyrene
10 microplate in which the interior of each well is coated with a thin layer of polystyrene-based scintillant.)

It will thus be seen that the present invention is directed to a method and material for carrying out rapid, enzyme based assays. The assays may be quantitative or qualitative, and do not require extensive sample preparation or
15 extraction steps. The assays rely upon the use of an amphipathic substrate which is reactive with the enzyme. The amphipathic substrate includes a hydrophilic portion which carries a reporter moiety (also referred to as a label), and a hydrophobic portion.

In a typical assay, a sample of material, including the suspected target
20 enzyme, is placed in contact with the hydrophobic layer, which as previously noted is preferably disposed upon a support. The sample is disposed in a polar solvent which typically comprises water, and at least a portion of the enzyme which is in the sample will interact with the substrate material therein. This interaction results in a cleavage of the substrate, which releases a hydrophilic fragment including the

reporter material thereupon. The hydrophilic fragment has a low affinity for the hydrophobic layer, and migrates into the exterior, polar solvent. Detection of the reaction is most preferably accomplished by measuring the residual reporter activity of the hydrophobic layer, and this residual reporter activity will be inversely proportional to the activity of the enzyme in the sample. Alternatively, enzyme activity may be measured by detecting the reporter label fragments in the aqueous material.

As noted hereinabove, the reporter moiety may comprise a radioactive species such as tritium, and in some instances, the hydrophobic layer may be disposed upon a support which includes a scintillator material therein. In other instances, the reporter material may comprise a magnetically labeled material, a fluorescent labeled material, a member of an antibody/antigen pair, a fluorescent agent, a color forming agent, a chemiluminescent material, an enzyme different from the target enzyme of the assay, an electrochemically active material, or the like, and other such reporter materials will be apparent to one of skill in the art.

The following examples are intended to illustrate the invention.

Example I

Sphingomyelinase Assay

Preparation of Substrate - FlashPlates coated with streptavidin were obtained from NEN Life Science Products™. Biotinylated phosphatidylethanolamine (PE) (Avanti) was diluted to 10 micrograms/mL in PBS (0.1M NaCl in 0.01M sodium phosphate buffer, pH 7.4) and 0.1 mL was added to each well. After incubation overnight at room temperature to allow binding to the streptavidin, the remaining solution was removed from the wells by aspiration. Sphingomyelin (egg), [choline

methyl- ^3H] (NEN Life Science Products, Inc.) was diluted to 1.0 microcurie/mL with Tris-HCl, pH 7.0 containing 0.1% bovine serum albumin [BSA] and 0.1 mL was added to each well. During overnight incubation at room temperature, the sphingomyelin hydrophobically interacted with the PE to form a ^3H -labeled solid-phase lipid layer. Plates were then aspirated and air-dried.

Enzyme Assay - Sphingomyelinase (Sigma) was serially diluted in PBS containing 1 mg/mL CaCl_2 and 1 mg/mL MgCl_2 (BioWhittaker) and 0.1 mL was added to each well and incubated overnight at room temperature. The hydrophilic ^3H -labeled fragment cleaved from the sphingomyelin by the activity of sphingomyelinase moved from the hydrophobic solid-phase lipid layer into the aqueous solution, decreasing the amount of ^3H within sufficient proximity of the scintillant to be counted. The results in Table 1 show that increasing the amount of sphingomyelinase produced a decrease in the amount of radioactivity detected in the well. The well with no sphingomyelinase was used as a negative control.

Table 1

Sphingomyelinase (mU/mL)	Counts per Minute (CPM)	Percent of Negative Control
2000	1038	8.6
200	2144	17.7
20	4672	38.6
2	8933	73.8
0.2	10691	88.4
0	12099	100.0

Example II

Phospholipase C Assay

Preparation of Substrate - FlashPlates were prepared exactly as in Example 1 except that PIP₂, [inositol-2-³H(N)] (NEN Life Science Products) was added instead of sphingomyelin-[³H] in the preparation of a ³H-labeled solid-phase lipid layer.

Partial Purification of PLC - (Camps et al. 1992; Camps et al. 1990; Glorschik et al. 1989). For maintenance, HL-60 cells (ATCC CCL-240) were cultured in 1-liter spinner flasks at 10% CO₂ in Iscove's Medium supplemented with 20% fetal bovine serum (FBS), 1% L-glutamine and 0.1% gentamicin. To induce phospholipase C, the cells were cultured in media supplemented with 1.25% dimethylsulfoxide (DMSO) for five days, or until a density of about 2-3 x 10⁶ viable cells was reached. After harvesting by centrifugation and washing twice with wash buffer (20 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM dithiothreitol, 3 mM benzamidine, 1 mM leupeptin, 1 mM phenylmethyl sulfonyl fluoride (PMSF) and 0.002 mM soybean trypsin inhibitor), the final pellet containing about 2 x 10¹⁰ cells (30 mL packed cell volume) was resuspended in 50-100 mL of Lysis Buffer (250 mM sucrose, 20 mM Tris-HCl [pH 7.5], 1.5 mM MgCl₂, 1 mM ATP, 3 mM benzamidine, 0.001 mM leupeptin, 1 mM PMSF, 0.002 mM soybean trypsin inhibitor). The cells were lysed by four treatments in the cold for one minute each with a Polytron homogenizer with a one minute rest between treatments, followed by sonication in an ice bath four times for one minute with a one minute rest between treatments. The lysate was supplemented with EGTA to 1.25 mM, and debris was removed by a 4°C centrifugation at 1445 x g for twenty minutes. The

resulting supernatant was centrifuged at 4°C for twenty minutes at 17,593 x g. The supernatant from this step was centrifuged at 4°C for sixty minutes at 112,594 x g. The final supernatant (cytosol) was passed through a 0.45 micrometer pore size filter. After determination of the protein concentration, the cytosol was snap
5 frozen and stored at -80°C, until used as a source of phospholipase C.

Enzyme Assay - To assay phospholipase C activity, the thawed HL-60 cytosol was diluted to various protein concentrations with PBS containing 0.4 mM CaCl₂ and 0.21 mM GTP-gamma-S. Diluted cytosol (0.1 mL) was added to each FlashPlate® well containing a solid-phase lipid layer with PIP₂-[³H], and incubated
10 overnight at room temperature. The hydrophilic ³H-labeled fragment cleaved from the PIP₂ by the activity of phospholipase C moved from the hydrophobic solid-phase lipid layer into the aqueous solution, decreasing the amount of ³H label within sufficient proximity of the scintillant to be counted. The results in Table 2 show that increasing the amount of phospholipase C produced a decrease in the
15 amount of radioactivity detected in the well. The well with no phospholipase C was used as a negative control.

Table 2

Phospholipase C (mg/mL of HL-60 cytosol protein)	Counts per Minute (CPM)	Percent of Negative Control
3.5	864	31.1
1.0	1072	38.5
0	2782	100

Example III

Chemical Surface Modification of FlashPlates for Covalent Linkage of Phosphatidylethanolamine

Two types of chemistries are involved: one employing divinylsulfone
5 (DVS) and the other using 1-3-(3-dimethylaminopropyl)carbodiimide (EDAC).

Divinylsulfone Plate Activation: A 1% DVS solution is prepared in 0.1 M sodium carbonate, pH 11.5, immediately prior to use. Aliquots (0.2 ml) are distributed to individual wells and the plate allowed to stand at room temperature for at least thirty minutes. The plate is then washed thrice with 0.1 M sodium
10 carbonate, pH 10.5, and then air dried briefly prior to adding the lipid. A 25 µg/ml solution of phosphatidylethanolamine (PE) is made in 25-50 mM sodium carbonate, pH 9.8-10.2 and 0.2 ml distributed to each well. The plate is incubated again at room temperature for at least three hours. Finally, the plate is washed twice with phosphate buffered saline (PBS), 0.04% deoxycholate and twice with
15 PBS alone. The plate is then air dried and stored in a sealed bag in the dark at room temperature.

EDAC Plate Activation: A solution of 0.5 mg/ml EDAC is prepared in 10-50 mM MES, pH 6.8-7.2, containing 25 µg/ml of PE and 0.2 ml is distributed to individual wells. The plate is allowed to stand overnight at room temperature in
20 the dark. Finally, the plate is washed twice with phosphate buffered saline (PBS), 0.04% deoxycholate and twice with PBS alone. The plate is then air dried and stored in a sealed bag in the dark at room temperature.

Introduction:

Inositol specific phospholipase Cs (PLCs) are key enzymes in the signal transduction of many cell mediated responses as diverse as peptide hormones and neurotransmitters (Rhee and Choi, 1992). These enzymes are specific hydrolases for the phosphoinositide. The enzyme reaction of the PLCs with phosphatidylinositol 4,5-bisphosphate (PIP₂) results in the production of inositol 1,4,5-trisphosphate (IP₃) and diacylglycerol. It is the generation of these products that produces the cascade of regulatory signals throughout the cell. IP₃ induces the intracellular release of Ca²⁺ stores from the endoplasmic reticulum through its specific interactions with the IP₃ receptor, whereas, diacylglycerol is a potent activator of protein kinase C. The activation of these cellular events results in a stimulation of a series of pathways that modulate cellular activity.

Classically, PLC enzymes are divided into three families designed β , δ and γ . The β and γ are regulated either through the G-protein coupled pathway or the receptor mediated tyrosine kinase pathway, respectively. Activity of these enzymes has been historically studied using solution based assays with phospholipid vesicles containing trace amounts of [³H, inositol] PIP₂ (DeVivo, 1994). The hydrolytic reaction is monitored by the addition of acidified organic solvents and subsequent phase separation. [³H] IP₃ remains in the aqueous phase while the acidic labeled lipids partition into the organic layer. Thus, simple radioassays of the aqueous layer provide a convenient way to measure PLC activity. However, the limits of the assay are that it is not readily amenable to high-throughput screening (HTS) methods.

In order to study these enzymes in high throughput fashion, a method was developed for monitoring PLC activity on FlashPlates™ containing bound [³H] phosphatidylinositol 4,5-bisphosphate. The FlashPlate™ was modified in order to capture labeled phospholipids onto the surface and, thereby, provide a functional substrate to gauge PLC activity.

Methods:

Phospholipid modified FlashPlates™ were made by a variety of different methods in order to test various coating surfaces. Ninety-six well Phospholipid FlashPlates™ were coated with 0.2 ml of a solution containing [1-³H, inositol] PIP₂ (~20 Ci/mmol, NEN) at concentrations between 0.25-1 µCi/ml for at least three hours. The wells were then washed twice with phosphate buffered saline (PBS) containing 0.04% sodium deoxycholate and finally with PBS alone. Typically, coefficient of variation ranged between 8-12%. Reactions were conducted directly in the wells in buffer indicated in the legends and monitored on a Packard TopCount™ instrument.

One unit of PLC activity is defined as one nmol of [³H] IP₃ formed per minute per mg of enzyme using typical solution based assays (DeVivo, 1994).

HL60 cells were grown to late log phase prior to harvest and the cytosolic fraction prepared as previously described (Camps et al. (1990; Camps et al. 1992) or HL60 cytosolic extracts were purchased commercially from ABS, Inc. A431 human epidermoid carcinoma cells were grown in 150 cm² culture flasks containing Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (FCS) prior to harvesting. A431 cells were cultured until ~50% confluency was reached. Cells were harvested by scraping the attached cell mass from the bottom of the

flask and pelleting by low speed centrifugation. Cell pellets were then rinsed twice with PBS. Cells were resuspended in a minimal volume of reaction buffer containing 10 units/ml streptolysin S and counted prior to being aliquoted to individual wells.

5 ~~All other chemicals or reagents were of reagent grade or better.~~

Results:

Our initial work served to verify that the labeled surface of the Phospholipid FlashPlate™ could effectively present the inositol headgroup to the enzyme for hydrolysis. Over the course of the 17.5 hour reaction purified
10 recombinant PLC γ 1 displayed an ability to release bound counts in a concentration dependent fashion (see Figure 1). In all instances the reaction of PLC γ 1 plateaued within the first five hours of the reaction and produced very little release of counts thereafter. At the highest concentration of enzyme tested, 130 μ g, over 75% of the radioactivity was released from the plate surface. These results indicated that
15 the majority of the surface bound [3 H] PIP₂ is readily available as a substrate for the enzyme. In order to verify that the release of surface bound counts was in fact due to hydrolysis of the [3 H] PIP₂ individual wells were aspirated and extracted with two volumes of chloroform-methanol under acidic conditions (CHCl₃: CH₃OH:HCl, 2:1:0.01). The relative distribution of radioactivity in the organic and
20 aqueous phase represents labeled PIP₂ and IP₃, respectively. As shown in Table 3, release of radioactivity from the labeled Phospholipid FlashPlate™ was due predominantly to the hydrolysis of the bound [3 H] PIP₂ with both crude PLC preparations from HL60 cytosolic extracts and recombinant PLC (control wells showed little or no release of radioactivity, data not shown). In both instances, the

radioactivity was distributed mostly into the aqueous phase (~90% and above) indicative of [^3H]IP₃. This result shows that labeled IP₃ was being specifically released from the Phospholipid FlashPlate™ and that surface bound labeled PIP₂ was in fact a viable substrate for both crude enzymatic PLC preparations of HL60
 5 cytosolic extracts, as well as the purified recombinant PLC.

Table 3

Post-reaction Organic Extraction of Aqueous Samples from
Individual Wells of [^3H] PIP₂ Coated Phospholipid FlashPlate™

Sample (# of experiments)	% Distribution	
	Aqueous Phase ([^3H]IP ₃)	Organic Phase ([^3H] PIP ₂)
HL60 cytosol (n=10)	89±7.5%	11 ± 1.1%
Recombinant PLC (n=3)	97±0.3%	3 ± 0.15%

The reaction also displayed many of the same characteristics as had previously been shown for solution based assays using mixed micelles in the
 15 presence of either deoxycholate (Crooke and Bennett, 1989) or spermine (Haber et al., 1991). For these assays recombinant PLC $\delta 1$ showed enhanced hydrolytic activity in a concentration dependent manner upon addition of sodium deoxycholate (see Figure 2A) or in the presence of added spermine (see Figure 2B). The results confirm the effects of these compounds on the stimulation of PLC
 20 activity as had been previously published (Crooke and Bennett, 1989; Haber et al. 1989) and validate the utility of the plate format when compared to solution based micelle assays.

Other experiments were designed to explore whether the stimulation of PLC activity from crude cellular extracts could be monitored on the Phospholipid

FlashPlate™ using previously described protocols (Rhee and Choi, 1992; DeVivo 1994). Various protein concentrations of HL60 cytosolic extracts were treated in the presence or absence of the non-hydrolyzable analog GTPγS and the decrease of radioactivity was monitored kinetically. As shown in Figure 3, in the absence
5 of the analog the release of bound counts displayed a concentration dependence upon the amount of protein added and the reaction began to taper off by three hours at ~15%. Addition of GTPγS to the samples produced a stimulation in PLC activity at every protein concentration tested. However, the concentration dependence of added protein appeared to be lost possibly due to the fact that only
10 low substrate levels were available on the surface. Even despite this, a clear 2-3 fold stimulation was evident for each protein concentration used.

As a further test of this format, we analyzed the activity of PLC as released from permeabilized A431 human epidermoid carcinoma cells (see Figure 4). In this instance, addition of various amounts of cells to the [³H] PIP₂ coated Phospholipid
15 FlashPlate resulted in the release of radioactivity in a cell number dependent manner. Cell numbers at and below 2 x 10⁵ cells/ml showed an initial lag phase on the reaction indicating a delay in the release of PLC activity from the cells. Moreover, at cell numbers about 2 x 10⁵ cells/ml the release of radioactivity from the surface could be shown to be due to the release of both intact lipid and IP₃,
20 (data not shown). Therefore, at elevated cell numbers this result suggests that other cellular components are capable of removing the lipid from the surface in a non-hydrolytic reaction. Cumulatively these results demonstrate that PLC activity can be monitored from both permeabilized cells and cytosolic extracts in a high throughput fashion on [³H] PIP₂ Coated Phospholipid FlashPlates™.

Conclusions:

This work clearly demonstrates the utility of [³H] PIP₂ coated phospholipid FlashPlates™ for monitoring PLC activity for high throughput screening of receptor-coupled functional assays. The results indicate that PLC activity can be monitored easily from a variety of sources including purified recombinant enzyme preparations to crude cell lysates and permeabilized cells. Moreover, this format provides a surface comparable to that used for classical labeled micelle studies and illustrates the feasibility of this assay for measuring PLC activity in a variety of different drug screening assays.

The principles of the present invention may be adapted to a variety of enzyme/substrate systems all of which will be apparent to one of skill in the art. In view of the foregoing, it will be understood and appreciated that numerous modifications and variations of the aforescribed invention may be readily implemented. The discussion, description and examples set forth herein are illustrative of particular embodiments of the present invention, but are not meant to be limitations upon the practice thereof. It is the following claims, including all equivalents, which define the scope of the invention.

Literature Cited

Camps et al. (1992) *Eur. J. Biochem.* 206:821-831.

Camps et al. (1990) *Biochem. J.* 271:743-748.

Crooke and Bennett (1989) *Cell Calcium*, 10:309-23.

De Vivo (1994) Assays for G-protein-regulation of phospholipase C activity. *Methods in Enzymology* 238:131-141.

Gierschik et al. (1989) *Eur. J. Biochem.* 183:97-105.

Haber et al. (1991) *Arch. Biochem. Biophys.*, 288(1):243-6.

Hianik et al (1996) Immobilization of enzymes on lipid bilayers on a metal support allows study of the biophysical mechanisms of enzymatic reactions. *Bioelectrochemistry and Bioenergetics* 41:221-225.

Hoekstra and Duzgunes (1993) Lipid mixing assays to determine fusion in liposome systems. *Meth. Enz.* 220:15-32.

Miller and Racker (1976) *J. Membr. Biol.* 26:319.

Mueller et al. (1962) *Circulation* 26:1167.

Ottova and Tien (1997) Self-assembled bilayer lipid membranes: from mimicking biomembranes to practical applications. *Bioelectrochemistry and Bioenergetics* 42:141-152.

Rhee and Choi (1992) *J. Biol. Chem.*, 267(18):12393-6.

Wright and Huang (1992) Bilayer stabilization of phosphatidylethanolamine by N-biotinylphosphatidylethanolamine. *Biochim. Biophys. Acta* 1103:172-178.

Claims

1 1. A method for analyzing a sample for the presence and/or activity of
2 an enzyme, said method comprising the steps of:
3 providing a hydrophobic material;
4 providing an amphipathic substrate having a hydrophobic portion, and a
5 hydrophilic portion, said hydrophilic portion including a reporter moiety thereupon,
6 said substrate being capable of being cleaved by said enzyme so as to produce a
7 hydrophilic fragment which includes said reporter moiety;
8 disposing said substrate in said hydrophobic material;
9 contacting the hydrophobic material having said substrate disposed therein,
10 with said sample and with a polar solvent, whereby any of said enzyme which is
11 present in said sample will cleave said substrate and produce said labeled
12 hydrophilic fragment, which fragment will migrate into said polar solvent; and
13 detecting the presence of said reporter in said polar solvent or in said
14 hydrophobic layer; whereby the presence of said reporter in said polar solvent
15 and/or the reduction of the quantity of said reporter in said hydrophobic layer is
16 indicative of activity of said enzyme.

1 2. A method as in claim 1, wherein said hydrophobic material
2 comprises a layer.

1 3. A method as in claim 2, wherein the step of detecting the presence
2 of said reporter comprises detecting the amount of said reporter in said
3 hydrophobic layer; wherein the concentration of said reporter in said hydrophobic
4 layer is inversely proportional to the activity of said enzyme.

1 4. A method as in claim 1, wherein the step of detecting the presence
2 of said reporter comprises detecting the amount of said reporter in said polar
3 solvent; whereby the concentration of said reporter in said polar solvent is
4 proportional to the activity of the enzyme.

1 5. A method as in claim 1, wherein the step of providing a
2 hydrophobic material comprises the further step of supporting said hydrophobic
3 material on a support.

1 6. A method as in claim 5, wherein said support comprises a micro-
2 well plate.

1 7. A method as in claim 5, wherein said support comprises a plurality
2 of beads.

1 8. A method as in claim 5, wherein said reporter is a radioactive
2 material and wherein said support includes a radiation responsive material.

1 9. A method as in claim 5, wherein said reporter is a non-radioactive
2 material and wherein said support includes a responsive material.

1 10. A method as in claim 2, wherein said hydrophobic layer is
2 covalently attached to said support.

1 11. A method as in claim 2, wherein said hydrophobic layer is a lipid.

1 12. A method as in claim 11, wherein said lipid comprises
2 phosphatidylethanolamine.

1 13. An assay for analyzing a sample for the presence of an enzyme
2 therein, said assay comprising:

3 a body of a hydrophobic material;

4 an amphipathic substrate disposed in said hydrophobic material, said
5 amphipathic substrate including a hydrophobic portion which interacts with said
6 hydrophobic material so as to retain said substrate therein, and a hydrophilic
7 portion having a reporter moiety thereupon, said substrate being capable of being
8 cleaved by said enzyme so as to produce a hydrophilic fragment which includes
9 said reporter moiety.

1 14. An assay as in claim 13, wherein said body of hydrophobic material
2 is disposed upon a support.

1 15. An assay as in claim 14, wherein said hydrophobic material is
2 covalently attached to said support.

1 16. An assay as in claim 13, wherein said hydrophobic material is a
2 lipid.

1 17. An assay as in claim 16, wherein said lipid comprises
2 phosphatidylethanolamine.

1 18. An assay as in claim 13, wherein said reporter comprises a
2 radioactive material or a non-radioactive material.

Recombinant PLC γ 1 Activity
on [3 H]PIP₂ Coated Phospholipid FlashPlate™

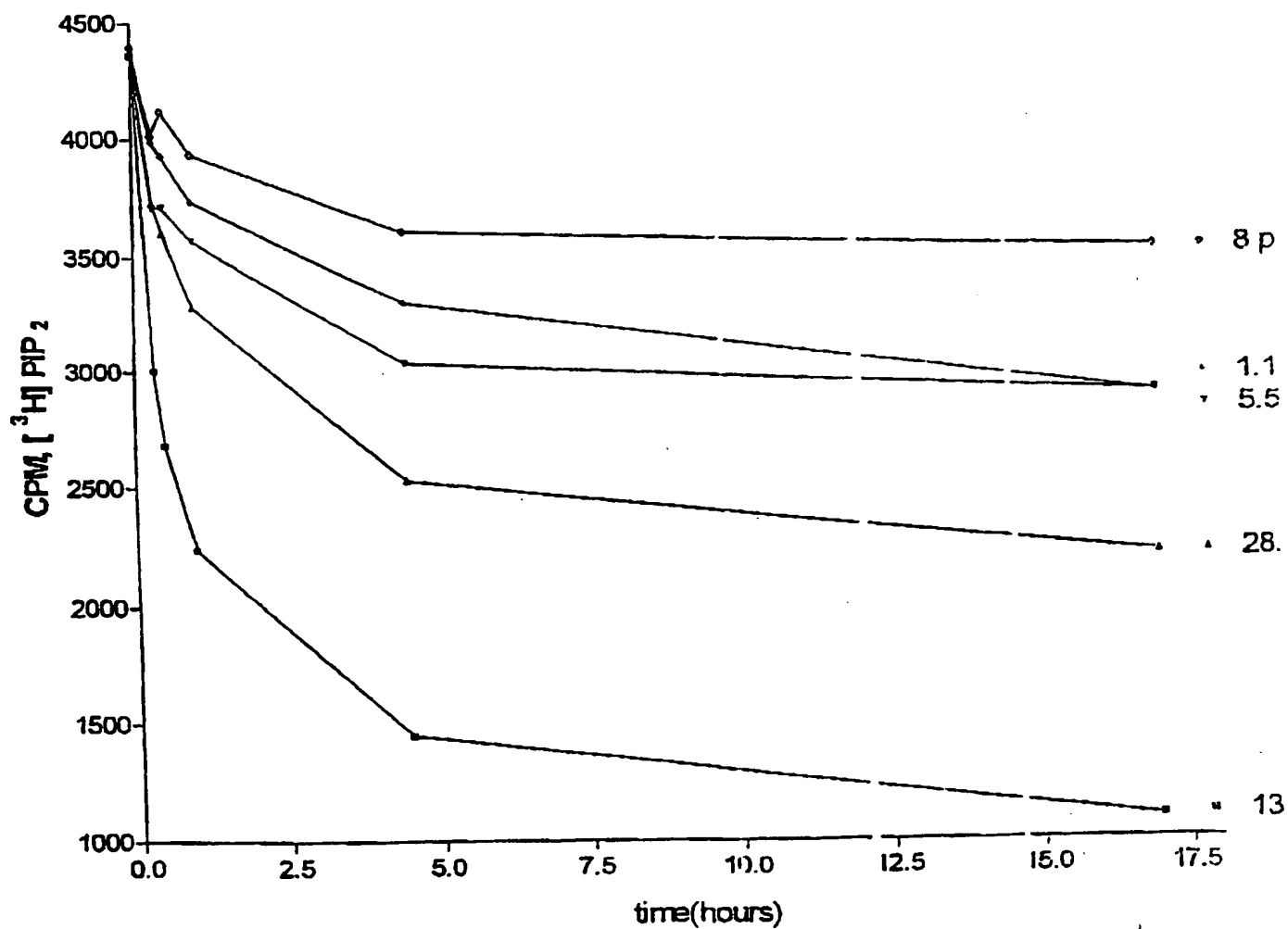


FIGURE 1

PLC $\delta 1$ Activity in the Presence and Absence of Additives on [^3H]PIP $_2$ Coated Phospholipid FlashPlateTM

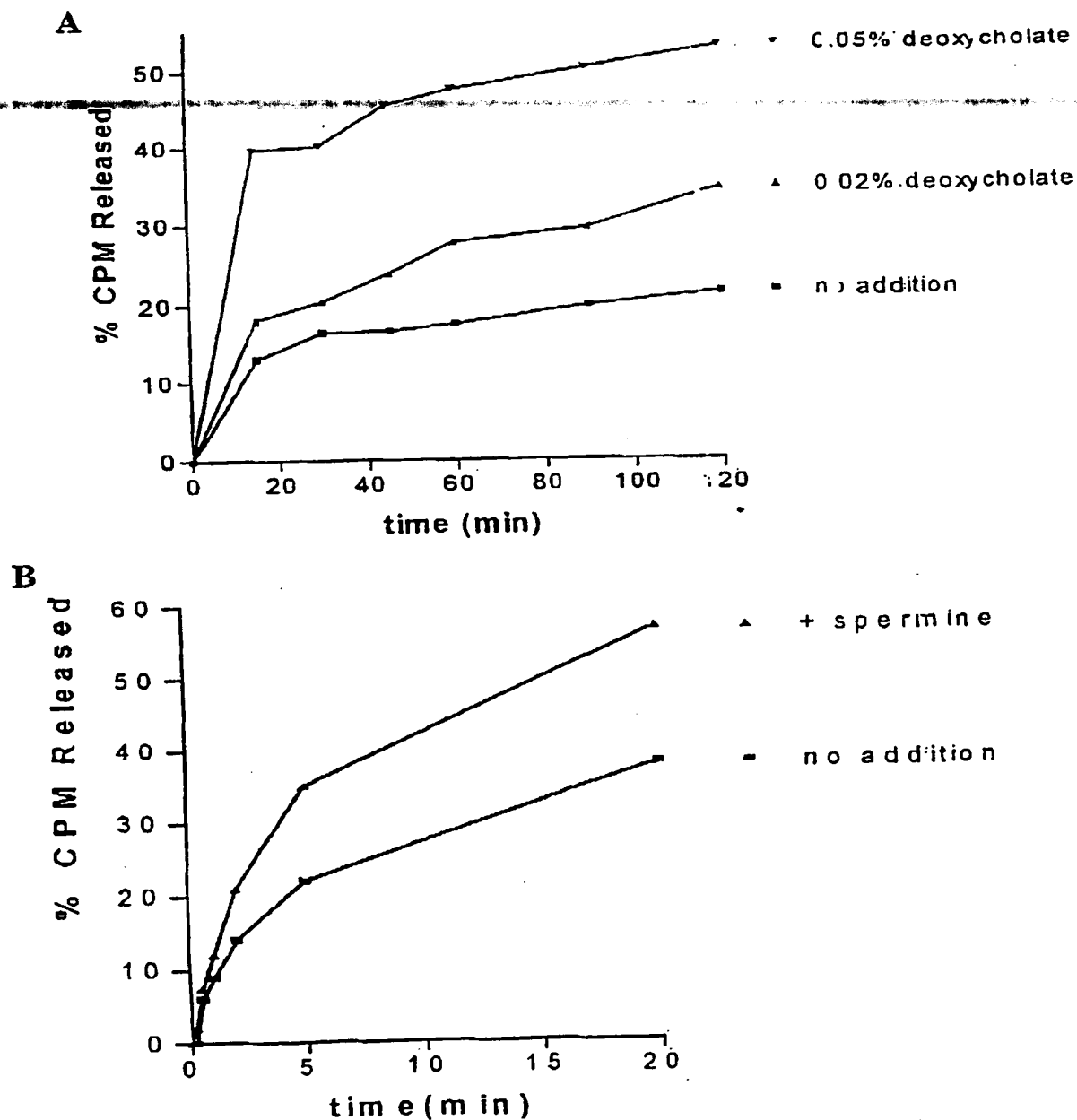


FIGURE 2A-B

PLC Activity of HL60 Cytosol \pm GTP γ S
on [3 H] FIP $_2$ Coated Phospholipid FlashPlate $^{\text{TM}}$

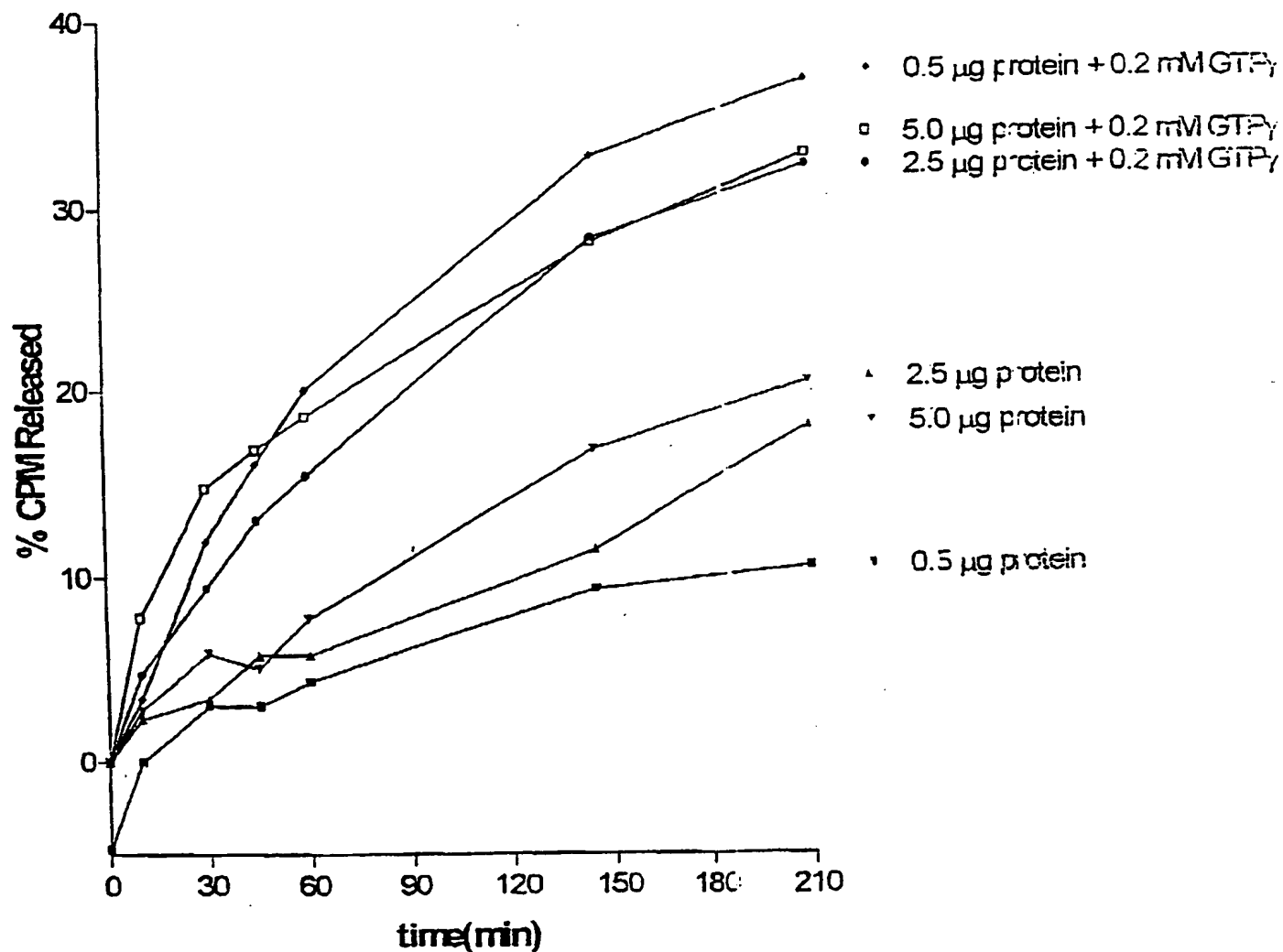


FIGURE 3

**PLC Activity of Permeabilized A431
Carcinoma Cells
on [³H] PIP₂ Coated Phospholipid FlashPlate**

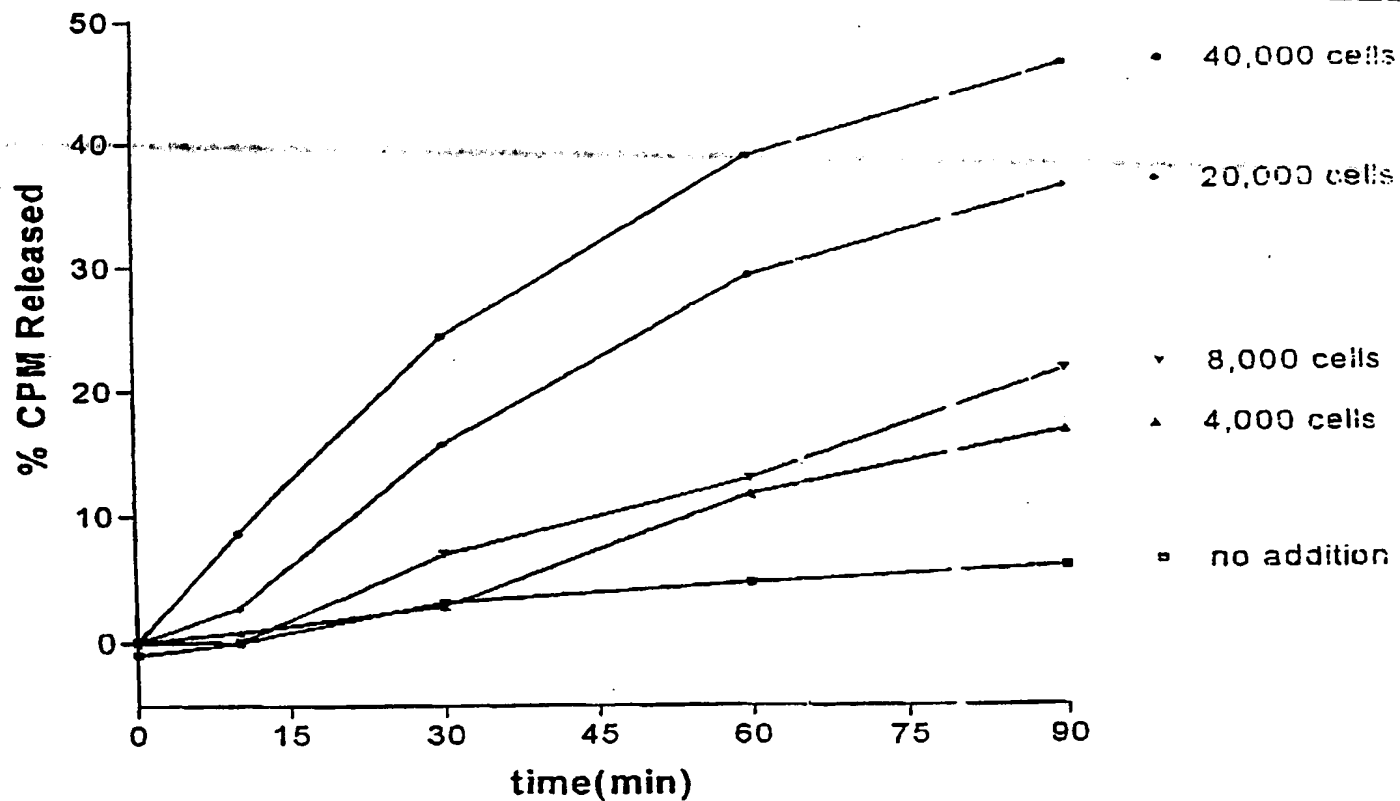


FIGURE 4

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/26818

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : C12Q 1/00;; G01N 33/53

US CL : 435/4, 968

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/4, 968

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
NONEElectronic data base consulted during the international search (name of data base and, where practicable, search terms used)
APS: ENZYME, PHOSPHATIDYLEHTANOLAMINE, HYDROPHOBIC

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 4,668,623 A (KINNUNEN et al) 26 May 1987, col. 9-10.	1-18

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
B earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*A* document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means	
P document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

15 MARCH 1999

Date of mailing of the international search report

29 MAR 1999

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

LOUISE LEARY

Telephone No. (703) 308-1235



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C12Q 1/00, G01N 33/53	A1	(11) International Publication Number: WO 99/32655
		(43) International Publication Date: 1 July 1999 (01.07.99)

(21) International Application Number: PCT/US98/26818

(22) International Filing Date: 17 December 1998 (17.12.98)

(30) Priority Data:

60/068,244	19 December 1997 (19.12.97)	US
09/212,211	16 December 1998 (16.12.98)	US

(71) Applicant: NEN LIFE SCIENCE PRODUCTS, INC. [US/US];
549 Albany Street, Boston, MA 02118 (US).

(72) Inventors: KASILA, Patricia; 16 Kent Street, Windham, NH 03087 (US). MULLINAX, Thomas, R.; 178 Quinobequin Road, Newton, MA 02468 (US). BEMBENEK, Michael, E.; 243 Mill Street, Burlington, MA 01803 (US). BOBROW, Mark, N.; 11 Battle Green Road, Lexington, MA 02421 (US).

(74) Agents: CITKOWSKI, Ronald, W. et al.; Gifford, Krass, Groh, Sprinkle, Anderson & Citkowski, P.C., Suite 400, 280 N. Old Woodward Avenue, Birmingham, MI 48009-5394 (US).

(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

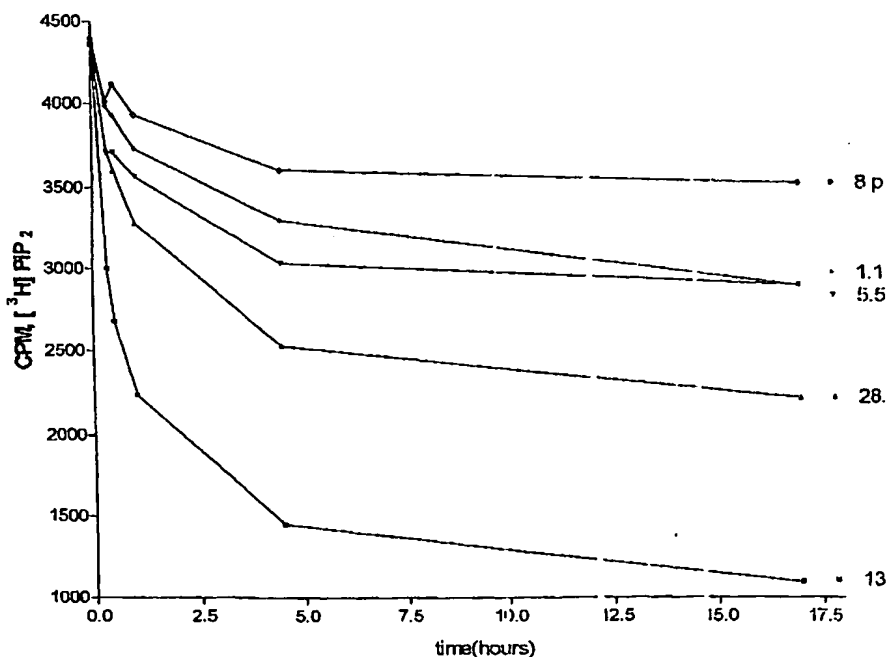
Published

With international search report.

(54) Title: AN ENZYME ASSAY USING A SOLID PHASE SUBSTRATE

(57) Abstract

This invention relates to a hydrophobic layer formed on a solid support, the hydrophobic layer incorporating by hydrophobic interactions an amphipathic enzyme substrate labeled with a reporter on its hydrophilic region, and the use of the same to conduct various enzymes assays without extraction steps. The figure is a graph illustrating kinetic activity of various amounts of purified recombinant PLC gamma 1 on [3H]PIP coated 96-well phospholipid FlashPlate (tm).

Recombinant PLC γ 1 Activity
on [3H]PIP₂ Coated Phospholipid FlashPlate™

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						